

THE RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND THE  
RESPONSE OF BLOWFLIES TO TARSAL STIMULATION  
BY ALIPHATIC ACIDS

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INTRODUCTION

A method for measuring the response of flies to tarsal stimulation by volatile organic substances was described by us in a previous report, in which data were given for the rejection thresholds of 15 aliphatic alcohols and a correlation established between the chemical structure of the compounds and their effectiveness in stimulation (1). The technique has now been extended in a study of the response to the aliphatic acids. For discussions of the results of earlier workers who have investigated the reactions of a variety of other organisms to this series of compounds the reader is referred to the reviews by Crozier (2), Cole (3), and Davson and Danielli (4).

As in our work with the alcohols, the antennae and labella were removed from 1 to 3 day old flies (*Phormia regina* Meigen) which were suspended from glass rods and offered the test solutions in an ascending series of concentrations in 0.1 M sucrose. Contact of the tarsi with an acceptable solution elicited an extension of the proboscis, and the minimum concentration of test substance which would prevent this response was recorded as the threshold for rejection. Flies were from a standard culture reared at 25°C. and 70 per cent relative humidity, while the tests were run in the laboratory on the day following operation at temperatures varying between 25° and 30°C. The solutions were made up carefully with double distilled water, commercial sucrose, and the following grades of acids:

Acid	Grade
Formic.....	Eastman 98-100 per cent M. P. 6-8°
Acetic.....	Eastman 99.9 per cent acetic acid
Propionic.....	Eastman B. P. 140-142°
n-Butyric.....	Eastman B. P. 161-163°
n-Valeric.....	Eastman B. P. 184-187°
iso-Valeric.....	Eastman B. P. 173-175°
Chloroacetic.....	Merck reagent M. P. 61-63°
Trichloroacetic.....	Merck reagent
Lactic.....	U. S. P. Mallinckrodt 85-90 per cent lactic acid
Pyruvic.....	Eastman
Glyceric.....	C. A. F. Kahlbaum, Berlin
Oxalic.....	Baker analyzed
Malonic.....	Eastman M. P. 134-135°
Succinic.....	Merck reagent

Acid	Grade
Malic.....	Eastman, <i>l</i> -malic acid
<i>d</i> -Tartaric.....	U. S. P. Merck
Fumaric.....	Eimer and Amend pure
Maleic.....	Eastman M. P. 138-139°
Hydrochloric.....	Baker analyzed 35-37 per cent hydrochloric acid
<i>iso</i> -Caproic.....	Eastman B. P. 110-111°/25 mm.
Stearic.....	Baker U. S. P.
Boric.....	Merck reagent 99.5 per cent boric acid

In correlating rejection thresholds for acids with molecular structure, it is necessary to recognize that in such experiments the receptors are confronted with a complex mixture of ionic and molecular species, as when they are stimulated with solutions of other electrolytes, and that none of the constituents should be excluded arbitrarily from consideration. The solutions offered in these tests contained, in addition to undissociated water and sucrose, hydrogen and hydroxyl ions, one or more species of anions, and undissociated acid. The problem was then to determine which components were effective and how much each contributed to the net result.

The flies were kept negative to plain water and positive to 0.1 M sucrose during the tests, and it was assumed as a working hypothesis that the attractiveness of the sugar was not altered by the presence of the other constituents in the test solutions. This assumption is open to question, for Yonge (5) has shown that the rate of passage of glucose through fresh membranes prepared from the foregut of the lobster was influenced perceptibly by the pH of the solution and went through a minimum at a pH of about 5.0, close to the isoelectric point of the cuticular intima. Ions other than H<sup>+</sup> also seemed to have specific effects in certain ranges. We have not found similar information relative to sucrose, nor is it known to what extent the properties of the cuticular covering of the tarsal receptors may resemble those of the gut of *Homarus*, but clearly the possibility of such changes in the strength of the attractive stimulus must be kept in mind in any discussion of rejection thresholds. Since the test substances in our experiments regularly gave pH values with the glass electrode which were practically identical with those computed from their dissociation constants for pure water, the presence of 0.1 M sucrose seems to have had little effect on their activities in solution. Also, the range of pH covered by the mean rejection thresholds was relatively narrow (roughly from 1.4 to 3.0), so that we believe it probable that variations in the attractiveness of 0.1 M sucrose have had little to do with the results obtained.

The concentration of hydroxyl ions in the test solutions was, of course, very much less than  $1 \times 10^{-7}$  M, and consequently this species may reasonably be disregarded in comparison with the hydrogen ions, anions, and undissociated acid, all of which were present in much greater concentrations. From the work of Frings (6) with the roach and of Frings and O'Neal (7) with *Tabanus*, it is

known that the hydrogen ion is more stimulating than the hydroxyl ion and that, for a series of salts with a common anion, it is the most stimulating of the cations. But it cannot be the sole factor in stimulation by acids, for in that case all should have been rejected at equal pH values. Inasmuch as this did not occur, it is evident that some other component,—either anions, undissociated acid, or both,—plays a part in stimulation.

TABLE I  
*Rejection Thresholds of *Phormia* for Fatty Acids*

Acid	Mean rejection threshold molar concentration $\pm \sigma m$	No. of flies tested	pH	pR—	pHR—	pHR	pK <sub>1</sub>	pK <sub>2</sub>
Formic.....	0.047 $\pm$ 0.0031	81	2.55	2.55	—	1.35	3.75	—
Acetic.....	0.26 $\pm$ 0.0048	61	2.67	2.67	—	0.59	4.74	—
Propionic.....	0.13 $\pm$ 0.0036	88	2.89	2.89	—	0.89	4.88	—
<i>n</i> -Butyric.....	0.082 $\pm$ 0.0027	59	2.96	2.96	—	1.09	4.82	—
<i>iso</i> -Valeric.....	0.066 $\pm$ 0.0027	74	2.98	2.98	—	1.19	4.77	—
<i>n</i> -Valeric.....	0.069 $\pm$ 0.0032	50	2.98	2.98	—	1.17	4.80	—
Chloroacetic.....	0.066 $\pm$ 0.006	60	2.04	2.04	—	1.24	2.85	—
Trichloroacetic...	0.020 $\pm$ 0.0016	50	1.74	1.74	—	2.77	0.70	—
Lactic.....	0.091 $\pm$ 0.0037	94	2.46	2.46	—	1.06	3.86	—
Pyruvic.....	0.062 $\pm$ 0.0043	50	1.90	1.90	—	1.31	2.49	—
Glyceric.....	0.112 $\pm$ 0.0078	61	2.31	2.31	—	0.97	3.65	—
Oxalic.....	0.059 $\pm$ 0.0057	50	1.53	2.94	1.57	1.68	1.42	4.31
Malonic.....	0.050 $\pm$ 0.0048	55	2.08	3.89	2.09	1.38	2.79	5.68
Succinic.....	0.106 $\pm$ 0.007	50	2.57	4.07	2.60	0.99	4.18	5.55
Malic.....	0.081 $\pm$ 0.0038	50	2.24	3.67	2.28	1.12	3.40	5.05
<i>d</i> -Tartaric.....	0.039 $\pm$ 0.0027	79	2.17	3.22	2.27	1.48	2.96	4.16
Fumaric.....	0.051 $\pm$ *	85	2.15	3.36	2.21	1.35	3.00	4.52
Maleic.....	0.037 $\pm$ 0.004	50	1.79	4.19	1.79	1.76	1.82	6.59
Hydrochloric.....	0.040 $\pm$ 0.0018	69	1.40	1.40	—	—	—	—

\* A saturated solution of fumaric acid was 0.051 M and only 50 per cent of the flies tested rejected at or below this level.

By an extension of this reasoning it is apparent also that the hydrogen ion concentration at rejection threshold is an inverse measure of the strength of the other contributing factor or factors. For if it be agreed that the intensity of stimulation at the rejection threshold is a constant, then for those substances in which a large proportion of the stimulus is furnished by the hydrogen ion a smaller proportion must come from other components. From this viewpoint the most stimulating acids are those which are rejected at the highest pH values, irrespective of the quantity in moles per liter which must be dissolved to achieve the result.

## RESULTS

The results are summarized in Table I, in which we have listed the mean rejection thresholds of the acids together with the standard errors and the number of flies used in each test. Also included are the negative logarithms of the calculated concentrations of hydrogen ions (pH), anions (pR<sup>-</sup> and pHR<sup>-</sup>), and undissociated acid (pHR), as well as those of the dissociation constants (pK<sub>1</sub> and pK<sub>2</sub>). The degree of dissociation of the acids,  $\alpha$ , was calculated from the approximation

$$\alpha = \sqrt{k/c} - (k/2c),$$

where  $k$  is the dissociation constant and  $c$  the molar concentration. The values of the dissociation constants were obtained from the papers of Dippy (8), Dippy and Jenkins (9), the International Critical Tables (10), and the Handbook of Chemistry and Physics (11). Routinely the pH values of the test solutions were determined with the glass electrode and found to agree within the error of measurement with those calculated.

In addition to the acids listed in Table I, flies were offered saturated solutions in 0.1 M sucrose of *iso*-caproic (0.04 M), stearic (0.001 M), and boric (0.8 M) acids, with the following results: 23 out of 31 flies accepted *iso*-caproic, all of 50 flies accepted stearic, and 36 out of 43 accepted boric.

On the basis of chemical structure, the organic acids in the table may be grouped into six series, each of which contributed certain salient facts to the correlation between chemical structure and stimulative effect.

*Monocarboxylic Acids*

With the saturated fatty acids from acetic through valeric the concentrations required for rejection decreased with increasing molecular weight. Formic acid was too stimulating, on the basis of concentration alone, to fit into its expected place at the beginning of this series, yet it falls into line if its greater degree of dissociation is taken into account. That is, when the several acids are compared in respect to pH at mean rejection threshold, rather than concentration, the order is: formic, acetic, propionic, *n*-butyric, *n*- and *iso*-valeric (see Table I). This result shows that the stimulating power of the acids, apart from their capacity for producing hydrogen ions, increases with increasing chain length. However, when the pH at rejection threshold is plotted against number of carbon atoms or such properties as boiling point or oil-water distribution coefficients, the relationship is non-linear on both arithmetic and logarithmic grids, and it is found that the increment in stimulative effect grows smaller as the series is ascended. An attempt was made to determine the rejection thresholds for *iso*-caproic and stearic acids, but saturated solutions of these in 0.1 M sucrose were acceptable to more than 50 per cent of the flies tested. These solutions should have been rejected had the rate of

increase in stimulating power with increasing molecular weight been as great as was found for the lower members of the series.

#### *Dicarboxylic Acids*

Results basically similar to the above were obtained with the three dicarboxylic acids, oxalic, malonic, and succinic, which form an orderly series when compared in respect to pH at rejection threshold, although the actual molar concentrations were 0.059, 0.050, and 0.106 respectively. Each of these acids had a lower pH at rejection than the corresponding monocarboxylic acid, which shows that the anions or free acid molecules of the dicarboxylic series are relatively less stimulating.

#### *Chloro-Substituted Acids*

As judged by the mean concentrations required for rejection, acetic acid is rendered considerably more stimulating by the introduction of one chlorine atom, and still more so by three. (Dichloroacetic acid was not available at the time of the tests.) But the greater stimulating power of the chloro-substituted acids is accounted for by their greater degree of dissociation, which results in a higher concentration of hydrogen ions relative to the amount of acid dissolved. The pH at rejection threshold was 2.67 for acetic, 2.04 for monochloroacetic, and 1.74 for trichloroacetic. Actually, then, substitution of chlorine for hydrogen reduces the stimulative power of the anion or free acid, but this is overshadowed by the large increase in degree of dissociation.

#### *Hydroxy Acids*

The effect of substitution of —OH for —H was investigated in two series of acids: propionic-lactic-glyceric ( $\text{CH}_3 \cdot \text{CH}_2 \cdot \text{COOH}$ ,  $\text{CH}_3 \cdot \text{CHOH} \cdot \text{COOH}$ ,  $\text{CH}_2\text{OH} \cdot \text{CHOH} \cdot \text{COOH}$ ), and succinic-malic-tartaric ( $\text{COOH} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$ ,  $\text{COOH} \cdot \text{CHOH} \cdot \text{CH}_2 \cdot \text{COOH}$ ,  $\text{COOH} \cdot \text{CHOH} \cdot \text{CHOH} \cdot \text{COOH}$ ). In both series the introduction of a single hydroxy group reduced the pH at rejection, and a further reduction was observed when a second hydroxy group was substituted. Evidently the replacement of —H by —OH lowers the stimulating power of the anion or free acid, and this must be compensated by an increase in concentration of hydrogen ions in order for stimulation to occur. Because of differences in the dissociation constants, these relationships are not invariably apparent from the concentrations of acid used in making up the solutions.

#### *Keto Acids*

Pyruvic was the only keto acid tested. It may be compared with propionic and lactic (= 2-hydroxypropanoic), which have the same number of carbon atoms. As noted above, substitution of an hydroxy group decreases the

stimulating effect of the anion or unionized acid and requires a compensatory increase in concentration of hydrogen ions. The results with pyruvic acid indicated that the stimulative power is weakened even more by the keto grouping, as shown by a comparison of the pH values at rejection, which were: propionic, 2.89; lactic, 2.46; pyruvic, 1.90.

#### *Unsaturated Acids*

Fumaric and maleic acids were tested as examples of compounds containing a double bond and also as a check on the influence of stereoisomeric configuration. The succinic-malic-tartaric series may be used for comparison. The pH of fumaric acid (= *trans*-butenedioic) at rejection was almost identical with that of *d*-tartaric (2.15 and 2.18). Thus the effect of the double bond is equivalent, approximately, to the introduction of two hydroxy groups. Maleic acid (= *cis*-butenedioic) was rejected only when a pH of 1.94 had been reached, on the average, but since it is more highly dissociated than fumaric, the molar concentration at rejection was somewhat less. Since the *cis*-form is chemically the less stable of the two, this comparison also suggests that the limiting process in tarsal stimulation is not the chemical interaction between the acids and some cellular constituent.

#### DISCUSSION

The results summarized above are in striking agreement with those reported for penetration of essentially the same groups of acids into the mantle cells of *Chromodoris* (12). This similarity provides a strong reason for believing that penetration of the receptors is the limiting process in tarsal stimulation, although, as we have pointed out elsewhere (1) it does not prove beyond doubt that penetration is an essential preliminary to sensory reception. The factors, presumably surface energy relationships, which favor penetration by many of the compounds in question would also favor their adsorption at a lipid-water or lipoprotein interface, and it is possible, in the absence of decisive evidence one way or the other, that simple accumulation of taste substances in the cell membrane could result in excitation.

In cases where penetration by acids is known to occur, there has been a good deal of discussion as to whether they enter the cell as ions or in the undissociated state, with perhaps the majority of writers upholding the latter alternative (4). But, as pointed out by Crozier (12), it is difficult to reconcile this concept with stimulation by the mineral acids, which are considered to be completely ionized in the concentrations required for sensory excitation. The results of the present study with the monocarboxylic series also are more readily understood in terms of penetration or accumulation in the ionic form, for the order of decreasing ionic concentrations at rejection threshold is that of increasing molecular weights, while no such correspondence is found when the series is

arranged in order of decreasing concentrations of free acid. Irregularities are revealed also in several of the other series when they are arranged in order of concentrations of free acid. (The data have been given in Table I.) Thus, with increasing chain length, the dicarboxylic acids show an increase in the amount of free acid at the rejection threshold. Among the hydroxy substituted acids, the monocarboxylic series shows first a decrease in free acid and then a rise as one and then two hydrogens are replaced, but the dicarboxylic series yields a decrease in free acid with both steps of hydroxy substitution. Now, it is true that all these differences could be reconciled by the assignment of appropriate arbitrary values for the stimulating power of the several free acid molecules, but it would seem more likely that the stimulating power changes in an orderly manner with successive similar changes in molecular constitution. Also, if the acids are acting mainly in the unionized form, it is difficult to see why the concentrations of free acid at rejection threshold in the monocarboxylic group do not form a Traube series, as is the case with the corresponding alcohols. For these reasons, pending the development of more direct evidence, we prefer the hypothesis that it is the anions, rather than the free acid molecules, which have furnished the second major component of tarsal stimulation.

Whether this detail of interpretation is eventually confirmed or disproved, it is quite apparent that the same general qualities have determined the relative effectiveness of the individual acids in all the series examined. As the affinity of the acid for water is increased, the stimulating power of the anion or free acid is diminished. This is true whether the structural change consists in removal of a  $\text{CH}_2$ -group, substitution of  $-\text{Cl}$ ,  $-\text{OH}$ ,  $=\text{O}$  or  $-\text{COOH}$ , replacement of a  $\text{C}-\text{C}$  linkage by a double bond, or a shift in stereoisomeric configuration (fumaric-maleic). Thus it is made clear that stimulation by the acids involves processes very similar to those inferred previously for the series of aliphatic alcohols, with the difference that in the case of the acids, the correlation between surface energy relationships and stimulatory power is obscured by the high effectiveness of the hydrogen ion. Since the tendency of the acids to dissociate commonly parallels their hydrophile character, weakening of the stimulative power of the anion or free acid molecule is accompanied by an increase in the ease of production of hydrogen ions. The actual effect on the receptors depends then on the balance struck between these two opposing factors.

#### SUMMARY

Using the technique of proboscis extension in antennectomized-labeledlectomized flies, the rejection thresholds of *Phormia regina* for 18 fatty acids and one mineral acid have been determined.

The conclusions reached on the basis of these data may be summarized in the following terms:

Tarsal stimulation by acids involves the summation of components from at least two sources. Of these the hydrogen ion is the most important. The other major factor is probably the anion rather than the undissociated acid. The stimulating power of the anions (or free acid molecules) increases with increasing chain length in both the mono- and dicarboxylic series, but the rate of increase decreases as the series is ascended. Acids containing 6 or more carbon atoms are not sufficiently soluble in 0.1 M sucrose to reach the threshold of rejection. Substitution of —H in the acyl grouping by —Cl, —OH, =O or —COOH, the presence of a C=C bond, or a shift from the *trans*- to the *cis*-configuration all diminish the effectiveness of the anion (or free acid). But since such alterations also augment the degree of dissociation and consequently the concentration of hydrogen ions, the net result is ordinarily a lowering of threshold in terms of the molar concentration required for rejection.

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